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Water Activity Studies with Total Phenols and Total Antioxidant Properties of Sun Dried, Grated and Stored *Vernonia amygdalina* Leaves

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ABSTRACT

Vernonia amygdalina leaves are widely utilized as food additive and for many herbal preparations in Nigeria. This study investigated the total phenols content and antioxidant properties of raw, processed and stored *Vernonia amygdalina* leaves. Processing methods used in this study entailed sun drying and grating. Storage conditions were in the open laboratory and at water activities (a_w) of 0.23, 0.52 and 0.97. All storages were carried out at ambient conditions and Storage duration was two months. All investigations carried out were in accordance with standard methods. The results indicated that in raw *Vernonia amygdalina* leaves, the total phenol content was 3.10±0.94mg/g and percentage antioxidant activity was 96.31±2.05%. It was further discernable from the results that processing and storage led to increases in total phenol contents, while there was a reduction of percentage antioxidant activity. Statistically, the storage changes observed with phenol determinations were at $P<0.05$ found to be significant. Furthermore, at $P<0.05$, the reduction in percentage antioxidant activity following processing and storage were statistically significant. These findings would be useful in the formulation of standard methods for the handling of *Vernonia amygdalina* leaves.

Keywords: Ambient, Antioxidant, Phenols, Spectrophotometer, *Vernonia amygdalina*, Water activity (a_w)

INTRODUCTION

Vernonia amygdalina is a perennial shrub from *Asteraceae* family (Sunmonu *et al*., 2022). The leaves are commonly referred to as bitter leaves. In Nigeria, the local names for *Vernonia amygdalina* as noted by Kokwaro (2009) are: *Ewuro* (Yoruba), *Onugbu* (Igbo), *Oriwo* (Bini), *Ityuna* (Tiv language), *Chusardoki or Fatefate* (Hausa language) and *Etidot* (Ibiobio). Furthermore, Kiguba *et al*. (2016) reported that *Vernonia amygdalina* in Uganda is called *Mululiza* and *Omubirizi*, in Ethiopia it is called *Ebichaa* and in Ghana *Vernonia amygdalina* is called *Awonwono*. The leaves of *Vernonia amygdalina* are widely used for ethnomedicinal and food additive purposes in Nigeria. As noted by Jarmai *et al.* (2022) *Vernonia amygdalina* has farvourable effects on health, which they attributed invariably, to its nutritional and phytochemical richness. Also, Ojimelukwe and Amaechi (2019) noted the presence of compounds with anticancer effects, antioxidant properties, antimalarial properties, anti-inflammatory properties, anti-bacterial characteristics and hypolipidemic benefits in *Vernonia amygdalina*. The therapeutic utilization of *Vernonia amygdalina* is well reported. Significantly, Igile *et al*. (1995) and Udochukwu *et al*. (2015) reported that a local infusion of the leaves or roots of the plant is used to treat microbiological infections, schistosomiasis, cough, hepatitis and other disorders that are spread through sexual activity. According to Okunlola *et al*. (2019) the roots and leaves of *Vernonia amygdalina* are used in traditional medicine to cure fever, hiccups and renal issues. It is reported by Njan *et al*. (2008) that the Medical Traditional Healer Association in Rukararwe, Uganda used *Vernonia amygdalina* to produce greenish powder packed in sachet and consumed as tea by patients suffering from malaria. Obviously, the pharmacological relevance of *Vernonia amygdalina* cannot be over emphasized.

Industrial utilization of *Vernonia amygdalina* is also been exploited. In particular, some fish farmers in Nigeria use liquid extract obtained from mashed and squeezed *Vernonia amygdalina* leaves as antibacterial agent in their culture farms. Furthermore, Adama *et al*. (2011) noted that the leaves of *Vernonia amygdalina* have been used in place of hops in the beer-brewing sector. Additionally, Yeap *et al*. (2010) reported that in Ethiopia, the honey wine called Tei is produced from bitter leaf. It is however imperative to mention that Ibrahim *et al*. (2009) posited that the use of *Vernonia amygdalina* is dose-dependent for successful results. There is therefore need for continuous investigations of the phytoconstituents of *Vernonia amygdalina*, especially, with respect to their responses to factors that could affect their level of occurrence in *Vernonia amygdalina*. The latter remark formed the basis of this work.

In Nigeria presently, *Vernonia amygdalina* leaves are stored after sun drying. Though this practice has helped sustained the availability of *Vernonia amygdalina* leaves especially during the dry season the aforementioned processing method and storage handling is unstandardized. There is need for standardization of the processing and storage handling of *Vernonia amygdalina* leaves, if considered from the point of view of safety and quality. It is imperative to mention that in its sun dried form, *Vernonia amygdalina* leaves are transported to wider locations. This makes it highly imperative for scientific documentations with respect to the effects of processing and storage on the compositional chemistry of processed and stored *Vernonia amygdalina* leaves to be well established. However, it would appear presently, that such documentations are scarce if any. There is need to fill this gap in knowledge, considering the high dependence on *Vernonia amygdalina* leaves as both food material and for its therapeutic values. Hence this study whose concern is to investigate in part, the influence of processing and storage on the total phenol and antioxidant properties of *Vernonia amygdalina* leaves is considered relevant; particularly, as diets are the main sources of human exposure to both nutritional and toxicologically relevant materials.

Total phenol and antioxidants could be relevant in managing the undesirable oxidative stress taking place in the human body. According to Sharafati-Chaleshtori, *et al*. (2011) phenolic compounds are a class of antioxidants, which act as free radicals terminators. This is particularly interesting as the reactive free radicals species have been noted by Pamplona – Roger (2005) to foster lipoprotein oxidation, arteriosclerosis, premature cellular aging, and even carcinogenic mutations. Furthermore, Solihab *et al*. (2012) also posited that phenols give protection against cardiovascular disease. Therefore, the less investigated total phenols contents of *Vernonia amygdalina* leaves, especially the aspects of their responses to processing methods and storage conditions, are worth investigating and reporting. Plant phenols can protect against lipoprotein oxidation (Hollman, 2001). Additionally, plant phenols are group of antioxidants that inhibit various stages of cancer process (Wattenberg, 1992). The relevance of this study can further be viewed from this perspective.

Storage conditions chosen for this study, will be in the open laboratory with samples kept in both opened and closed containers; as well as at separate water activity (a_w) of 0.23, 0.52 0.97. The concept of water activity is nowadays universally adopted by food scientists and technologists to quantify availability (Coultate, 2002). Additionally, Belitz *et al*. (2009) noted that the storage quality of food does not depend on the water content, but on water activity (a_w). The work of some other researchers (Acker 1969; Schoebel *et al*., 1969; Labuza *et al*., 1970; Lajollo *et al*., 1971; Eichner and Karel 1972; Ukhun 1984; Ukhun 1986; Ukhun and Uwatse 1988; Ukhun, and Dibie 1989; Ukhun and Dibie 1991; Dibie and Ukhun 2019; Dibie and Ukhun 2019; Dibie and Ukhun 2019; Dibie and Ukhun 2020; Dibie and Ukhun 2020) have also shown that food stability, safety and other properties can better be predicted from a_w than from water content. Water activity in particular, plays central role in food stability.

In this study spectrophotometric methods were used to quantify total phenols and total antioxidant properties of the samples. Furthermore, data that was generated has been statistically analyzed. Notably, aspects of descriptive statistical evaluation of data and statistical evaluation of the relation between variables (ANOVA) were carried out. International Business Machine (IBM), Statistical Package for Social Sciences (SPSS) was used in statistical evaluation of data.

MATERIALS AND METHODS

Sample Collection

Vernonia amygdalina leaves used in this study were obtained from a local farm in Iyowa town, via Benin City, Edo State.

Samples Inspection and Cleaning

The *Vernonia amygdalina* leaves were pretreated to free them from various forms of contaminants. In particular, they were those free from viral, bacteria or fungal infection. Also, contaminating plants and/or plants parts were identified and removed. Clearly in this study, only healthy *Vernonia amygdalina* leaves were used.

Samples Preparation

Fresh *Vernonia amygdalina* leaves were sun dried to constant weight and grated using Black and Decker 650W, BX550 blender. Subsequently, the grated samples were sieved, with the aid of a 16 – mesh standard sieve (Pascall Eng. Co. Ltd. Sussex, England).

Samples Storage

Air tight desiccators wherein a_w of 0.23, 0.52 and 0.97 were established in accordance with the method prescribed by Rockland (1960) were initially prepared. Subsequently, three hundred grams of sun dried and grated *Vernonia amygdalina* leaves were weighed in triplicates into different 500ml glass beakers (Pyrex glass), and kept in the separate air tight desiccators. Samples were stored for 2 months, and on monthly basis, they were investigated for the examined parameters. All the storage desiccators were kept on laboratory bench at ambient conditions.

Determination of Total Phenols Content

Total phenol was determined spectrophotometrically by the Folin – Ciocalteau method as described by Kujala *et al*. (2000), in which to 1ml of methanolic extract of sample and

standards (gallic acid solutions of concentration: 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10mg/l prepared by dissolving gallic acid in a 1:1, v/v mixture of methanol: water) in separate test tubes, were added 5ml each of Folin–Ciocalteau reagent (1:10 dilution with distilled water) and thoroughly mixed. Thereafter, 4ml of 1M Na_2CO_3 was added to each of the test tubes and again, thoroughly mixed. Subsequently, the solution was allowed to stand for 30min in the dark at room temperature. Blank was also prepared. This was followed by absorbance reading at 765nm, using Uv/visible spectrophotometer (Jenway spectrophotometer, 6715 Uv/vis). The total phenol content was calculated from the standard graph of gallic acid, and the results were expressed as gallic acid equivalent (mg/g), which is a common reference compound.

Determination of Total Antioxidant Activity

The free radical scavenging activity of the extracts was determined, using the 1, 1-diphenyl–1–picrylhydrazyl (DPPH) assay, in accordance with the method described by Liyana-Pathiranan, and Shahidi (2005). Ascorbic acid and gallic acid were used as reference standards and were dissolved in methanol. The concentration of test extract and standards used for the determination was 250µg/ml, obtained by serial dilution. To each of separate 2.5ml of methanolic plant extracts and standards in different test tubes were added 1.0ml of freshly prepared DPPH solution (5.9mg/100ml methanol). Thereafter, the respective reaction mixtures were incubated in the dark at room temperature for 30mins. Subsequently, the absorbance of the respective reaction mixtures was read at 517nm, using Uv-Visible spectrophotometer (Jenway spectrophotometer, 6715 Uv-Visible). Measurements were carried out in triplicates. A lowered absorbance value (greater discolouration) indicated higher radical scavenging activity. The percentage antioxidant activity was calculated, using equation 1.

$$\% \text{ Antioxidant Activity} = 100 - \left(\frac{\text{Abs sample}}{\text{n Abs control}}\right) \text{ x } 100 \quad (1)$$

Methanol was used as the blank.
Abs control = absorbance of DPPH radical + methanol.
Abs sample = absorbance of DPPH radical + sample extract.

The positive controls were the values obtained with the reference standards (ascorbic acid and gallic acids). The obtained percent antioxidant activity of the respective test extracts were compared with the positive control.

RESULTS AND DISCUSSION

Results for Quantitative Determination of Total Phenols

Results for quantitative determination of total phenols in raw, sun dried, grated and stored *Vernonia amygdalina* leaves are presented in Table 1. Findings indicate the presence of phenols in raw, processed and stored *Vernonia amygdalina* leaves. The reported occurrence of phenols in *Vernonia amygdalina* leaves as findings in this work indicate is consistent with the findings of Inusa *et al*. (2018). Some researchers including Luo *et al*. (2017); Ali *et al*. (2019); and Edo *et al*. (2023) in their works, also reported the occurrence of phenols in the *Vernonia amygdalina* leaves samples they investigated. It is desirable that phenols occur in *Vernonia amygdalina* leaves, as phenols retard oxidative degradation of lipids, and thereby improve the quality and nutritional value of foods, especially when in storage. Worthy of note also is the remark of Cowan (1999) that several studies have demonstrated the antimicrobial activity of phenols and /or phenolic extracts. In fact, some authors (Ankit *et al*., 2010; Adama *et al*., 2011) have earlier noted that *Vernonia amygdalina* could act as a bittering agent and a hop substitute used for controlling microbial contamination in beer brewing. It would appear therefore, that *Vernonia amygdalina* leaves especially the sun dried and stored form could be used as ingredients in certain food processing, where amongst other functions, they can supply natural antioxidants and perform antimicrobial functions.

Value obtained for quantitative estimation of total phenol in raw *Vernonia amygdalina* leaves was 3.10±0.94mg/g. The factors which influence the composition of foods of plant origin include genetic constitution, method of propagation, growing conditions, age or maturity at the time of harvest, as well as length and condition of storage before use (Joslyn 1970; Yamaguchi and Wu, 1975). Additionally, Kumar *et al*. (2017) noted that studies have shown that the nature and quantity of phytochemicals differ according to the season and geographical location. It would appear therefore, that the reported value of total phenols contents of samples of *Vernonia amygdalina* leaves used in the study could have been influenced by several factors.

It is discernable from results presented in Table 1, that sun drying of *Vernonia amygdalina* leaves led to increases in the total phenols contents of the studied samples. Additionally, results further indicate that storage of sun dried and grated *Vernonia amygdalina* leaves in the open laboratory under ambient conditions, fostered further biosynthesis of phenols in samples stored in both closed and opened containers. Also deducible from results presented in Table 1, was that at the end of the two months of storage, samples kept in the

closed containers had higher concentrations of total phenols, than the samples kept in the open containers. It would appear therefore, that when sun dried and grated *Vernonia amygdalina* leaves are stored in closed containers, the conditions therein promoted faster biosynthesis of total phenols, than when samples are kept in opened container and stored in the open laboratory.

Findings from this work have also shown that the various studied water activity (a_w) under which sun dried and grated *Vernonia amygdalina* leaves were stored, supported further biosynthesis of phenols in the stored samples. It was interestingly found that the highest increase in total phenols content was recorded in the samples stored at a_w 0.23 (Table 1). Significantly, the trend of results was such that with increasing a_w, the level of increases in total phenols decreased. Notably, the increases in total phenols content were lowest in the samples stored at the very high a_w of 0.97. What is discernable here is that the prevailing conditions at the low a_w of 0.23, favoured more of the series of reactions that led to increases in the total phenols levels in the studied stored samples, vis-à-vis the degradation reactions; far and above the conditions at the higher a_w of 0.52 and 0.97 also studied in this work.

The observation as noted in this study, that total phenols increased in value when sun dried and grated *Vernonia amygdalina* leaves are stored, is worthy of note; especially as sun drying of *Vernonia amygdalina* leaves is the commonest processing and/or preservation method. The implication here is that handlers of *Vernonia amygdalina* leaves should ensure that sun dried, grated and stored form of it remain nutritionally and therapeutically safe.

The reported storage increases in the values of total phenols in the stored and studied samples of sun dried and grated *Vernonia amygdalina* leaves, can also be explained even if in part, by considering the effects of the mechanical disintegration of the cell walls of the samples, following the grating operation. According to Ukhun (1984) in milled cowpea flour, physical attributes such as large surface area, high degree of porosity, enzyme decompartimentalization following milling and the milling operation which is a form of stress, promoted chemical responses. Thus, it could also mean that in the studied samples, grating of sun dried *Vernonia amygdalina* leaves prior to storing, fostered a wide array of chemical reactions, such as histological disintegration, and enhanced enzyme decompartmentalization usually related to grating operations. These events would have positively influenced the production and accumulation of phenols, in the stored samples.

Results of determinations of percentage antioxidant activities of raw, sun dried, grated and stored *Vernonia amygdalina* leaves extracts are presented in Table 2.

Findings from Table 2 indicate that the percentage antioxidant activity for *Vernonia amygdalina* leaves extract was 96.31±2.05%. According to Erasto *et al*. (2007) the leaves extract of *Vernonia amygdalina* scavenged 75-99.3% DPPH. The results obtained in this study, is consistent with their findings, as it is within the range reported by Erasto *et al*. (2007). The prevailing conditions with respect to whether the conditions are favourable or not, will to a large extent, determine the availability of the different species of antioxidants found in a particular plant. It is suggested therefore, that the reported percentage antioxidant activities of the examined *Vernonia amygdalina* leaves samples be viewed from this perspective. In this work, the reported antioxidant activity of *Vernonia amygdalina* leaves extracts is considered high. Significantly, the obtained percentage antioxidant activity of *Vernonia amygdalina* leaves extracts (96.31±2.05%) at the concentration of 0.25mg/ml, is higher than those of the standards (ascorbic and gallic acids) at the same concentration of 0.25mg/ml, in the DPPH free radical scavenging activity test. Therefore, the antioxidant property of *Vernonia amygdalina* leaves could be exploited as antioxidants source for industrial applications.

In *Vernonia amygdalina*,extracts, Igile *et al.* (1994); Ayoola *et al*. (2008); Farombi and Owoeye (2011) posited that the occurrence of flavonoids in them is responsible for their antioxidant property. Antioxidant factors in plants are numerous. Significantly, Alara *et al*. (2017) noted that in vivo biochemical analysis of *Vernonia amygdalina* leaf extracts on \rats showed an appreciable increase in the level of the antioxidants: superoxide, dismutase,, catalase,, glutathione and malondialdehyde. The combined presence of different antioxidants in plants would have synergistic effect in their actions. Thus, it could mean that synergism contributed to the overall percentage antioxidant activity of *Vernonia amygdalina* leaves which led to the high percentage antioxidant activity value obtained for *Vernonia amygdalina* leaves in this work. It is important that *Vernonia amygdalina* leaves possessed antioxidants activities, as *Vernonia amygdalina* leaves could be source of natural antioxidants to consumers and thus, help to maintain the body's antioxidants composition.

Table 1: Total Phenol Contents of Raw, Sun Dried, Grated and Stored *Vernonia amygdalina* Leaves Samples

S/N	Parameter	Raw (fresh) sample	Sun dried and pre-stored sample	Stored samples									
				Storage conditions/time (months)									
				a_w0.97		a_w0.52		a_w0.23		Open Laboratory			
										Covered container		Opened container	
				2-months	1-month	2-months	1-month	2-months	1-month	2-months	1-month	2-months	1-month
1	Phenols (mg/g)	3.10± 0.94	3.82±0.62	9.37 ± 1.40	4.11 ± 0.58	12.43 ± 1.71	6.82 ± 1.20	17.46 ± 3.18	10.39 ± 2.14	10.25 ± 1.81	4.59 ± 0.16	4.18 ± 1.10	3.99 ± 0.72

Table 2: Percentage Antioxidant Activity of Raw, Sun Dried, Grated and Stored *Vernonia amygdalina* (Bitter) Leaves Extracts

S/N	Storage Condition / Sample Description	Total antioxidant capacity (%) / Time (months)		
		Pre-storage	1-month	2-months
1	a_w0.23, stored sample extract (0.25mg/ml)	95.91 + 3.62	88.23 + 2.18	81.17 + 1.69
2	a_w0.52, stored sample extract (0.25mg/ml)	95.91 + 3.62	85.36 + 1.90	75.97 + 2.06
3	a_w0.97, stored sample extract (0.25mg/ml)	95.91 + 3.62	81.52 + 0.84	69.29 + 1.25
4	Open Laboratory (covered container), stored sample extract (0.25mg/ml)	95.91 + 3.62	83.44 + 1.64	80.94 + 3.19
5	Open laboratory (uncovered container), stored sample extract (0.25mg/ml)	95.91 + 3.62	77.69 + 2.10	62.93 + 0.55
6	Sun dried pre-stored sample extract (0.25mg/ml)	95.91 + 3.62	ND	ND
7	Raw sample extract (0.25mg/ml)	96.31 + 2.05	ND	ND
8	Ascorbic acid (0.25mg/ml)	93.61 + 4.15	93.41 +2.95	92.17 +1.55
9	Gallic Acid (0.25mg/ml)	91.74 + 1.88	91.68 + 0.74	90.25+1.02

ND = Not Determined

It is also deducible from results, that sun drying, grating and storage of *Vernonia amygdalina* leaves, led to reduction in their percentage antioxidant activity values. With respect to the effects of sun drying operation on *Vernonia amygdalina* leaves, possibly, some antioxidant species in this plant are photo-sensitive and were therefore adversely affected by sun drying. Additionally, it could also mean that some degradation reactions aided or even initiated by light, occurred during the sun drying of *Vernonia amygdalina* leaves. Apparently, these effects cumulatively, would lead to reduction in the percentage antioxidant activity of sun dried *Vernonia amygdalina* leaves. With respect to grating obviously, grating would create greater surface area for reactions to take place. Apparently, if the overall consequences of these reactions are degradative in nature, then grating of the sun dried *Vernonia amygdalina* leaves prior to storage should foster loss in some of the grated *Vernonia amygdalina* leaves constituents. It is pertinent to mention also, that the collapse of cell walls following grating, would promote contact between certain exogenous enzymes and some protected substances. Clearly, this could lead to enzymatic transformation of the protected substances into other forms; which subsequently, and probably, led to certain changes in their biological activities. Significantly, the contact of ascorbic acid with ascorbic acid oxidase will foster oxidation of ascorbic acid into substances with little or no reducing properties, for which ascorbic acid is known for. Instructively, ascorbic acid is one of the most powerful antioxidants provided by nature.

Also discernible from results is that in sun dried, grated and stored *Vernonia amygdalina* leaves samples, progressive storage losses with time, occurred in their percentage antioxidant activity values. This was observed in all the samples stored at the various storage conditions. It was further noted that the magnitude of reduced retention of antioxidant activity, varied with storage conditions. Significantly, with respect to samples stored in the open laboratory under ambient conditions, results indicate that those stored in opened containers suffered greater loss in antioxidant activity, compared to samples stored in closed containers. What is anticipated here is that continuous contact of the stored samples with atmospheric factors including oxygen and moisture, fostered greater loss in antioxidant activities of the sun dried, grated and stored *Vernonia amygdalina* leaves. This could mean that the loss in antioxidant activities of the stored samples proceeded via some oxidative and hydrolytic degradation processes.

Results from the portion of work on water activity studies with antioxidant activity of stored sun dried and grated *Vernonia amygdalina* leaves indicate that progressive antioxidant losses occurred with time; and the losses occurred more at the higher a_w. Several factors could have been responsible for this. Significantly, at the higher a_w increased amount of available water could foster solubilisation of water soluble antioxidant entities, with a subsequent increase in their rate of destruction. In stored cassava and garri, Ukhun and Dibie (1991) posited that increased levels of available water could have promoted increased oxygen dissolution in food materials, leading to increased oxidative loss of ascorbic acid. Thus, it would appear that increased oxygen dissolution particularly at the elevated a_w, fostered increase in oxidation reactions, which probably led to increased antioxidant species losses. The breakdown of crystalline regions of the samples following increase in levels of available water, should promote oxygen diffusion into them and would consequently, result in increased antioxidant species degrading reactions. According to Labuza (1972) elevated a_w may act to lower the activation energy for ascorbic acid destruction. Thus, it could also mean that for the stored samples, especially at the elevated a_w, lowering of the activation energy for various antioxidant species destructions occurred.

CONCLUSION

Findings from this work have shown that *Vernonia amygdalina* leaves contain phenols and also exhibited antioxidant activities. Additionally, results indicated that the processing and storage conditions investigated led to increases in total phenols levels. On the other hand, the processing and storage conditions investigated led to decrease in total antioxidant activities. Therefore, while the processing methods and storage conditions studied in this work could be used to enhanced total phenols levels in *Vernonia amygdalina* leaves, the same cannot be said with respect to antioxidant activities. Significantly, what this means is that in addition to phenols, *Vernonia amygdalina* leaves contain some other constituents with antioxidant activities, some of which however, were adversely affected by the processing methods and storage conditions studied in this work.

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